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Direct observation of mammalian cell growth and size regulation

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Abstract

We introduce a microfluidic system for simultaneously measuring single cell mass and cell cycle progression over multiple generations. We use this system to obtain over 1,000 hours of growth data from mouse lymphoblast and pro-B-cell lymphoid cell lines. Cell lineage analysis revealed a decrease in the growth rate variability at the G1/S phase transition, which suggests the presence of a growth rate threshold for maintaining size homeostasis.

The lack of consensus on how mammalian cells grow over generations^{1–7} may largely stem from technical limitations. Almost all prior studies of size homeostasis have monitored populations of cells. In a typical experiment, a population of cells would be synchronized in the cell cycle and then their average cell size monitored over time as the synchronized cells grew and eventually divided. Such experiments are limited not only by the poor resolution afforded by cell cycle synchronization and the unavoidable dispersion that follows, but also by artifacts produced by the synchronization methods themselves. Techniques for synchronization typically block nuclear division but not cell growth and inevitably result in oversized cells^{4,5}. In the most comprehensive single cell study yet to examine the interrelationship of cell growth and the cell cycle, single yeast cells were studied microscopically using a fluorescent reporter protein as a proxy for cell mass⁸. By correlating cell mass to specific cell cycle events, a cell size threshold for cell cycle entry was observed. Although protein content may be the dominant component within a cell, the use of a fluorescent reporter protein does not guarantee a precise readout of a cell's biomass. By

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AUTHOR CONTRIBUTIONS

S.S. and Y.W. developed methods for multi-generation cell growth measurements. A.T. transformed the L1210 cell line with FUCCL. S.S., A.T., P.J., M.W.K. and S.R.M. designed the experiments and analyzed the data. S.S. developed an optical system for simultaneous fluorescent measurement and performed the experiments. J.K. assisted with the data acquisition and cell culture. S.S., A.T., Y.W., P.J., M.W.K. and S.R.M. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

S.R.M. is a co-founder of Affinity Biosensors and declares competing financial interests.

contrast, advanced forms of microscopy for measuring cell dry mass have been applied to cell growth but they have generally suffered from limited precision⁹.

To overcome the technical problems that have limited the study of cell growth, we recently developed a direct approach for dynamically monitoring the mass of individual growing cells. We have demonstrated that this method, employing a suspended microchannel resonator (SMR) mass sensor, has the potential to weigh animal cells with a precision near 0.01%¹⁰. The SMR measures the buoyant mass of objects that pass through it. When an object that is denser than the water passes through the device, the net increase in mass (i.e. the buoyant mass of the object) lowers the resonant frequency. By continually measuring buoyant mass when the cell travels back and forth through the sensor, the growth of individual bacteria, yeast, and mammalian cells has been observed¹¹. However, the resulting continuous shear stress limited the growth duration to less than an hour, the method was not configured to observe cell cycle progression and uncertainty introduced by the fluidic control system effectively limited the buoyant mass precision to ~1%.

Here we describe three technological advancements for the SMR that enable precise measurement of cell growth and cell cycle progression. First, an individual cell and its progeny can be weighed over many generations. By storing the cell in a large bypass reservoir and only occasionally passing it through the SMR, the cell experiences only limited exposure to shear forces (Fig. 1a, Supplementary Video 1 and Online Methods). The interdivision time for single L1210 mouse lymphoblast cells in this new apparatus is similar to its doubling time in culture, suggesting that cell growth is unperturbed (Supplementary Fig. 1). Second, we have used a unique form of hydrodynamic focusing to confine the flow path of the cell as it travels through the SMR (Supplementary Fig. 2). Without such focusing, the cell can wander in a direction orthogonal to the flow stream, which creates position dependent error. Hydrodynamic focusing enables the cell's buoyant mass and growth rate to be measured with a precision near 0.05% and 3%, respectively, which represents a ten-fold improvement over prior work¹¹. Third, we have integrated a microscope with the SMR so that a cell can be observed while it is stored within the bypass channel (Fig. 1a). This enables monitoring of cell cycle events using fluorescent ubiquitination-based cell cycle indicators (FUCCI)¹². In the experiments reported here, the L1210 mouse lymphoblast cells stably expresses fluorescently tagged proteins that are only present during early to late G1 phase of the cell cycle (Cdt1-mKO2, red fluorescence) or during late-G1/S/G2/M phases (Geminin-mAG, green fluorescence). Fluorescent signals are monitored concurrently with cell buoyant mass over multiple generations (Fig. 1b), enabling size and changes in growth properties to be linked to cell cycle position.

Because the growth of single cells has never been measured before with such precision, we could identify previously undescribed aspects of the growth trajectory. Most striking is the transition in growth rate that occurs mid-way through the cell cycle. During the first several hours after cytokinesis there is a rapid increase in growth rate, followed by a period where the growth rate increases more slowly (Fig. 1c), a behavior consistent with the prior population measurements on these cells¹³. Out of 122 cells measured, 50% showed a transition with a distinct change in growth rate, 20% showed a less distinct change in growth rate, while the remainder showed erratic growth trajectories (Supplementary Fig. 3). We wondered if the growth rate transition might coincide with the transition of cells out of G1 phase – which is thought to be devoted largely to growth – and into S-phase – where DNA synthesis occurs. The FUCCI signals were measured over the complete cell-cycle in 40 cells, of which 20 showed an obvious growth rate transition. There was a strong correlation between the growth rate transition and entry into S phase (Fig. 1c).

A unique benefit of single cell growth trajectories is the ability to register cell-to-cell variability (Fig. 2). Within each lineage of several cells, there was variability in the instantaneous growth rate of newborn cells that typically ranged from 2 to 4 picograms/hour, and these rates were independent of size (Fig. 2a). For a given lineage of cells, the variability in growth rate decreases as cells progressed through G1 and begins to increase following the G1/S phase transition. The cell size at this transition point varies between different sets of lineages (Supplementary Fig. 4). That newborn cells closely related in lineage have different initial growth rates, yet enter S phase with similar growth rates, suggests that there is a threshold in growth rate that gates the G1 to S phase transition. Even among independent lineages, the coefficient of variance of growth rate at birth decreased to about 60% at the G1/S phase transition (Fig. 2e). We also observe a reduction in size variation between birth and G1/S, although to a much smaller extent (1.7%). Furthermore, the growth rate-per-mass trajectories (Fig. 2a and Supplementary Fig. 4) saturates upon entry into S phase. The rapid increase in growth rate-per-mass in G1 indicates that the growth rate is not simply proportional to cell mass and suggests that there may be unique regulation mechanisms established during the G1 phase of the cell cycle.

Further support for a growth rate threshold for entry into S phase, is the strong negative correlation we observed between the duration of G1 and the growth rate in early G1 (Fig. 2b). Since all cells increase their growth rate in G1, slow growing newborn cells may achieve the growth rate threshold by spending more time in G1. This trend became even more pronounced as we decreased the growth rate of cells by limiting nutrients (Supplementary Fig. 5). Furthermore, contrary to previous experiments with yeast where small newborns tended to exhibit a prolonged G1 phase¹⁴, we found that neither the duration of G1 nor the interdivision time correlates with newborn cell size (Supplementary Fig. 6). This, together with the findings shown in Figure 2a, suggests that deterministic growth regulation¹⁵ can be based on a critical growth rate rather than a critical size.

To determine if a growth rate threshold at the G1/S transition occurs in other types of cells, we measured growth trajectories of the FL5.12 pro-B-cell lymphoid cell line. Supplementary Figure 7 shows a ~110 hour continuous buoyant mass measurement of 12 generations of cells from the same lineage of FL5.12 and an analogous measurement for L1210 cells for comparison. Similar to what we observed for L1210 cells, the variability in growth rate for FL5.12 cells decreases as cells progressed through G1 and then begins to increase following the G1/S transition (Fig. 2c). Also consistent with L1210 cells, there is a strong negative correlation between the G1 duration and growth rate in early G1 (Fig. 2d). Unlike L1210 cells, which showed no correlation between the duration of G1 or the interdivision time with newborn cell size, there were measurable but weak correlations in FL5.12 cells (Supplementary Fig. 6).

Cell growth and cell size are known to adapt to external conditions². To explore this in more detail, we grew L1210 cells in limiting isoleucine. Under these conditions, the growth rate was reduced and the duration of the G1 phase and interdivision time increased by 1.6-fold (Supplementary Fig. 8a). Despite this increase, the size range at both the G1/S phase transition and at cytokinesis remained virtually the same as cells in normal medium (Supplementary Fig. 8b, 9). Furthermore, the presence of a strong negative correlation between the initial growth rate and the length of G1 (Supplementary Fig. 5) and a reduction in the variability of the growth rate at the G1/S phase transition (Supplementary Fig. 10) suggests that a growth rate threshold is still present.

Measurement of individual growth trajectories with a precision sufficient to register cell-to-cell variability reveals that growth and the cell cycle are tightly coupled in more complex ways than previously imagined. First, contrary to earlier notions that growth is either

constant or simply a function of cell mass, there is a demonstrable, distinct change in growth rate at the G1/S transition, suggesting that growth and cell cycle are closely linked. Second, cells grown in nutrient poor media have the ability to maintain their size at the G1/S transition and cytokinesis by slowing cell cycle progression in proportion to their reduction in growth rate. Finally, the capacity of the SMR method to examine the trajectories of individual cells is what allowed us to conclude that the G1/S transition is not gated by a critical size threshold. Instead, the drop in growth rate variability suggests that the G1/S transition responds to a critical growth rate. Such a model could not be obtained without knowing the precise growth trajectories of individual cells. The critical role of a growth rate threshold in size homeostasis may not be as obvious a model as a size threshold. But there are several potential biochemical targets that could directly reflect the growth rate, and there are no known mechanisms in mammalian cells by which size itself could be sensed.

ONLINE METHODS

Suspended microchannel resonator (SMR)

As described in Ref 16, cells suspended in solution flow through the SMR, and the resulting frequency shift depends on the mass and position of the particles. When a cell passes through the SMR, this measurement yields a peak whose height is directly proportional to the cell's buoyant mass. SMR devices are fabricated by creating buried channels in silicon-on-insulator wafers, followed by wafer thinning and dry etching to form suspended microchannels with 2–3 μm thin walls and a 15 μm fluid layer. Two hundred devices are fabricated and vacuum-packaged on a six-inch wafer with yields exceeding eighty percent. A getter layer prevents slow degradation of the on-chip vacuum due to outgassing. Integrated under each cantilever is an electrostatic drive electrode and the cantilever vibration is detected by the optical-lever. A gain controlled oscillator circuit is used to continuously track the resonance frequency of the SMR during the growth measurement.

Single cell growth measurements

To achieve long-term growth measurements of single cells, an approach shown schematically in Supplementary Figure 11 was devised to culture the cell within the two bypass reservoirs adjacent to the SMR. Computer controlled pressure regulators with pressurized glass sample vials on vertical translational stages are used to precisely control fluid flow within the SMR chip. Every 30 to 60 seconds, feedback between the mass signal and pressure regulators causes the cell to transit from one bypass to the other within a period of approximately 1 sec. This process is fully automated and the specific sequence of steps used to capture and maintain the cell within the device is outlined in the caption of Supplementary Figure 11. Since the bypass reservoirs are optically transparent, the cell can be observed by a modular microscope (Supplementary Video 1). Upon division, both cells initially flow back-and-forth between the bypass channels. However, the feedback randomly selects the mass signal from one of the cells. As a result, the other cell slowly drifts away from the one in feedback and is eventually swept away in the bypass channels. To increase measurement precision, hydrodynamic focusing was used to minimize position dependent error within the SMR (Supplemental Fig. 2).

L1210 culture and FUCCI transformation

L1210 murine lymphoblasts (ATCC-CCL219) were adapted to Leibovitz's L-15 CO₂ independent media (Invitrogen) and maintained in media supplemented with 10% FBS (Invitrogen), 1g/L D-(+)-glucose solution (Sigma-Aldrich) and 1% 100X penicillin-streptomycin solution (Gemini). For growth measurements in media with limited isoleucine, L-15 media without any isoleucine was supplemented with 10% FBS. The FUCCI constructs mKO2 (monomeric Kusabira-Orange2)-hCdt1 and mAG (monomeric Azami

Green)-hGem were cloned into Lentiviral vectors carrying the neomycin or blasticidin resistance genes respectively¹⁷. Virus particles were generated by transfection of 293T cells followed by standard virus purification. L1210 cells were first infected (Polybrene 8 μ g/ml) with mAG-hGem carrying viruses. After selection (3 μ g/ml Blasticidin), cells were infected again with the mKO2-hCdt1 viral particle and selected using 400 μ g/ml of Geneticin to form the FUCCI L1210 line. Cells used in this study originated from a single cell.

Fluorescent measurement

A modular microscope (Nikon) was mounted on top of SMR and a 50x objective lens (Nikon-CFI Plan BD ELWD N.A 0.55, W.D 9.8mm) was used to collect the fluorescent signal into two separate photomultiplier tubes (H5784-02) (Supplementary Figs. 12 and 13). To measure red and green fluorescence simultaneously, wideband metal halide illumination (Lumen-200pro) was used with a dual-cavity dichroic mirror (Chroma-59004) and single cavity emission filters (red-ET585/40m, green-HQ520/20m both Chroma). Illumination was shuttered by the light source and two field-stops were used to confine the area of illumination and imaging to minimize the background noise. Fluorescent signals were measured at the collimated plane.

pH stability for FL5.12 culture

RPMI (Invitrogen) cell growth media used for FL5.12 cells requires 5% CO₂ for bicarbonate buffering. To achieve this, the media vials were pressurized with 5% CO₂ gas (Airgas), which stabilized the pH of media in the vial. However, due to the CO₂ leakage in the tubing and the gasket, dissolved CO₂ was lost from the media on the way to the chip and limited cell growth in the bypass channels. PEEK tubing (IDEX), which has minimum gas permeability, was used to reduce CO₂ leakage. Nevertheless, CO₂ leakage still occurred at the tubing-to-chip gasket interface. As a result, on-chip pH monitoring was required for determining the optimal flow conditions and device geometry for maintaining a stable pH. This was accomplished by utilizing the fluorescent indicator BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein), which is often used for monitoring intracellular pH. The pH sensitivity of BCECF in the SMR optical system was calibrated by using a plate reader (Tecan). Real-time monitoring in the bypass reservoir revealed that the pH would increase within 1–2 hours if the media was not replenished with sufficient frequency (Supplementary Fig. 14). Since the media replenishment rate is governed by the cell transit frequency (typically 1–2 times per minute), achieving stable pH came at the cost of exposing the cell to detrimentally high levels of shear. To overcome this, the SMR chip was designed with enlarged bypass reservoirs (cross-sectional dimensions were increased by two-fold to 1400 μ m² by 2800 μ m²) which enabled the replenishment rate to be increased by five-fold for the same transit frequency. As a result, a stable pH was observed for more than 15 hours when the cell transited the SMR as infrequently as once every 100 sec.

Data processing

To calculate the rate at which a cell accumulates biomass (growth rate) from measurements of buoyant mass versus time, the raw data (sampled at every 30 seconds) was smoothed by a 30 minutes moving average window and the time derivative was taken at each point. The buoyant mass and growth-rate measurement errors were determined as the standard deviation of the buoyant mass and growth rate measurements from either beads or fixed cells over acquisition periods greater than 12 hours. Three steps were used to find the location of the growth rate transition: i) A second order polynomial was fit to the initial buoyant mass versus time trajectory with progressively longer time windows. ii) The duration of the window for when the fitting error increased above a defined threshold was determined. iii) The size and growth rate at the end of the time window was then used in a bilinear fit as an initial value of the growth rate transition point. The bilinear fit of the

growth rate over buoyant mass provided four parameters: growth rate acceleration before and after the transition, the mass and growth rate at the transition point.

Supplementary Material

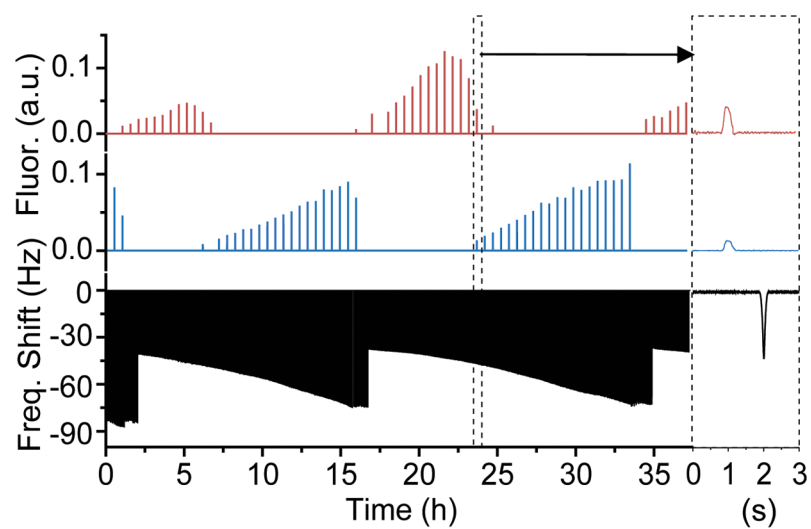
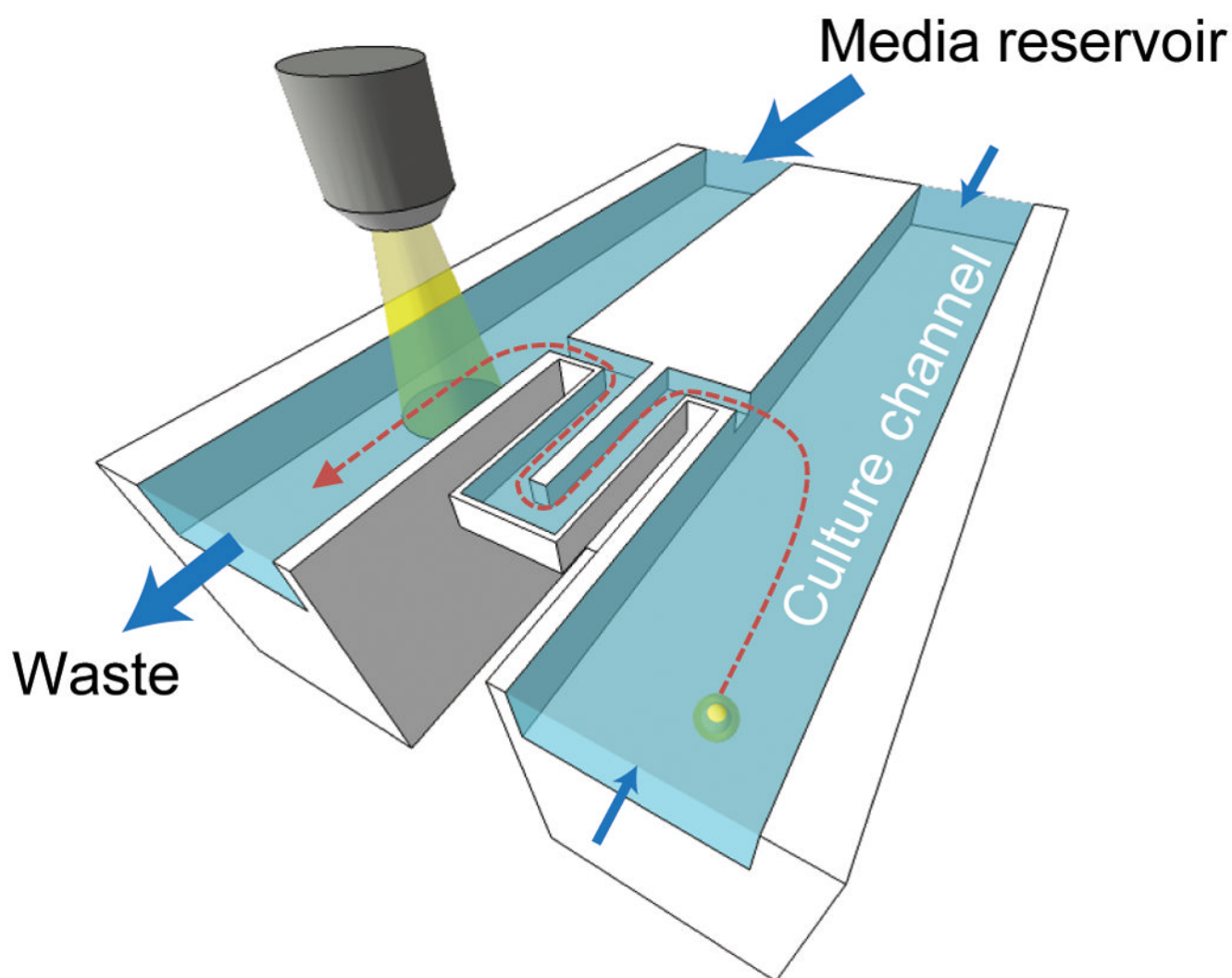
Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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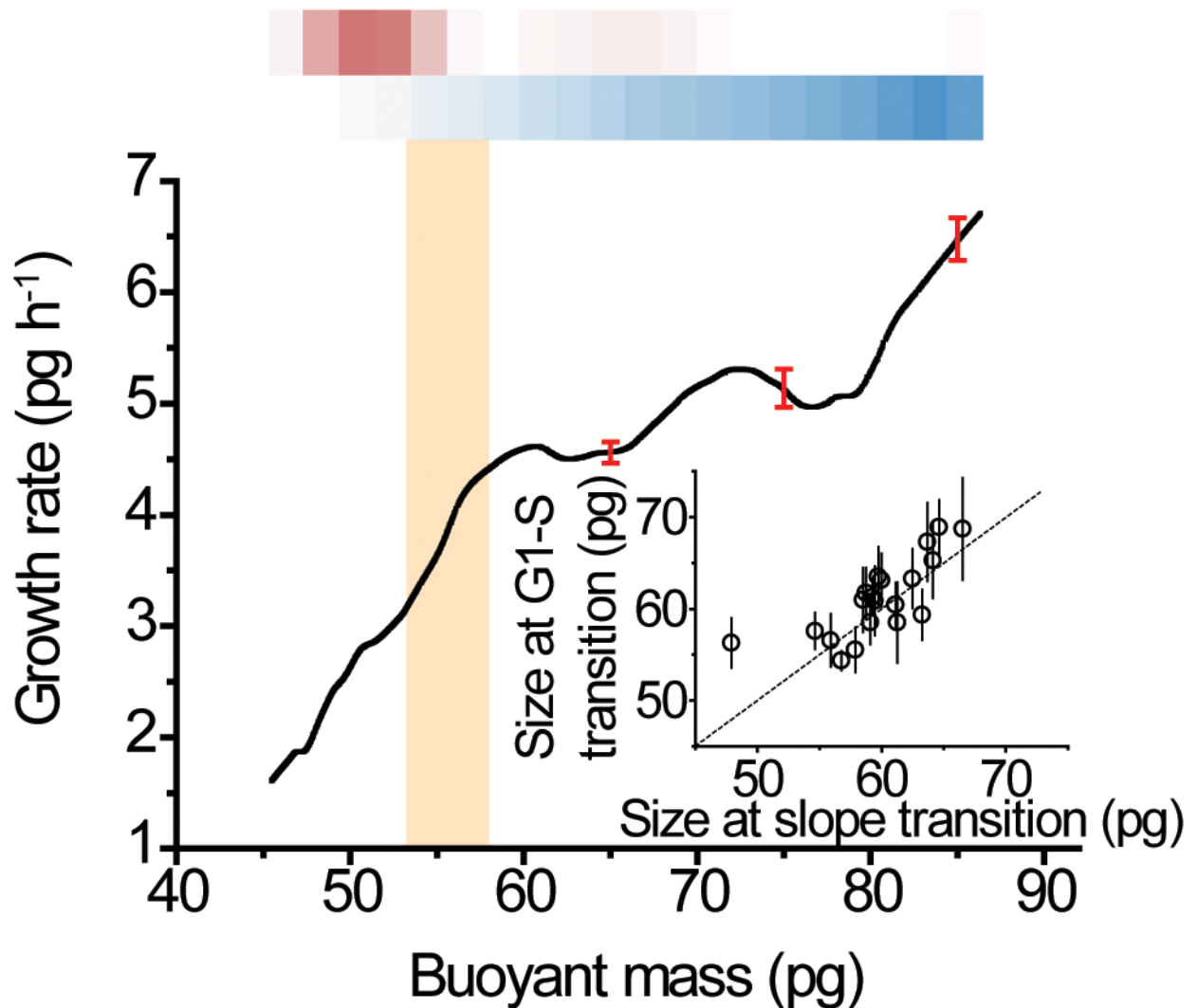
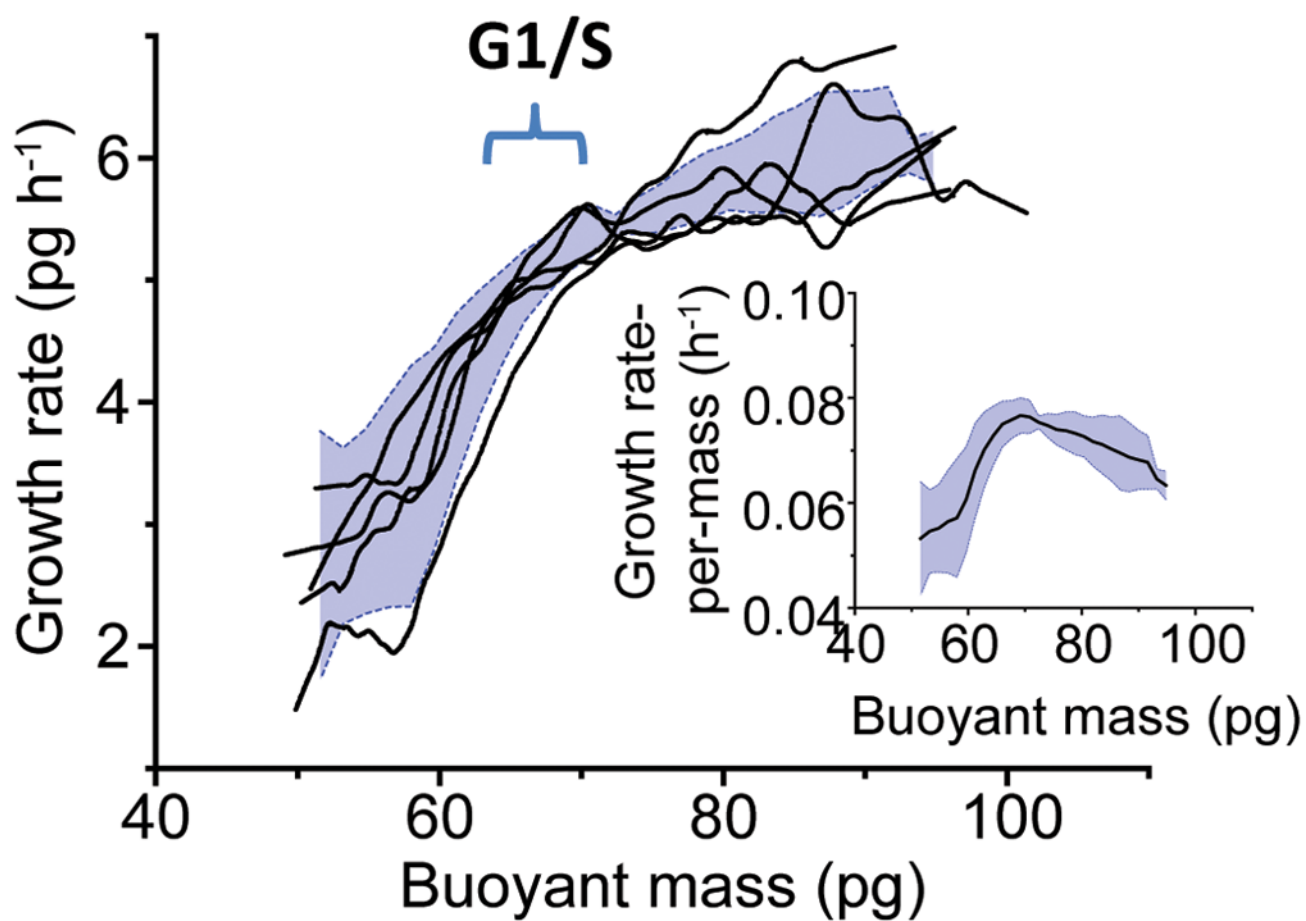
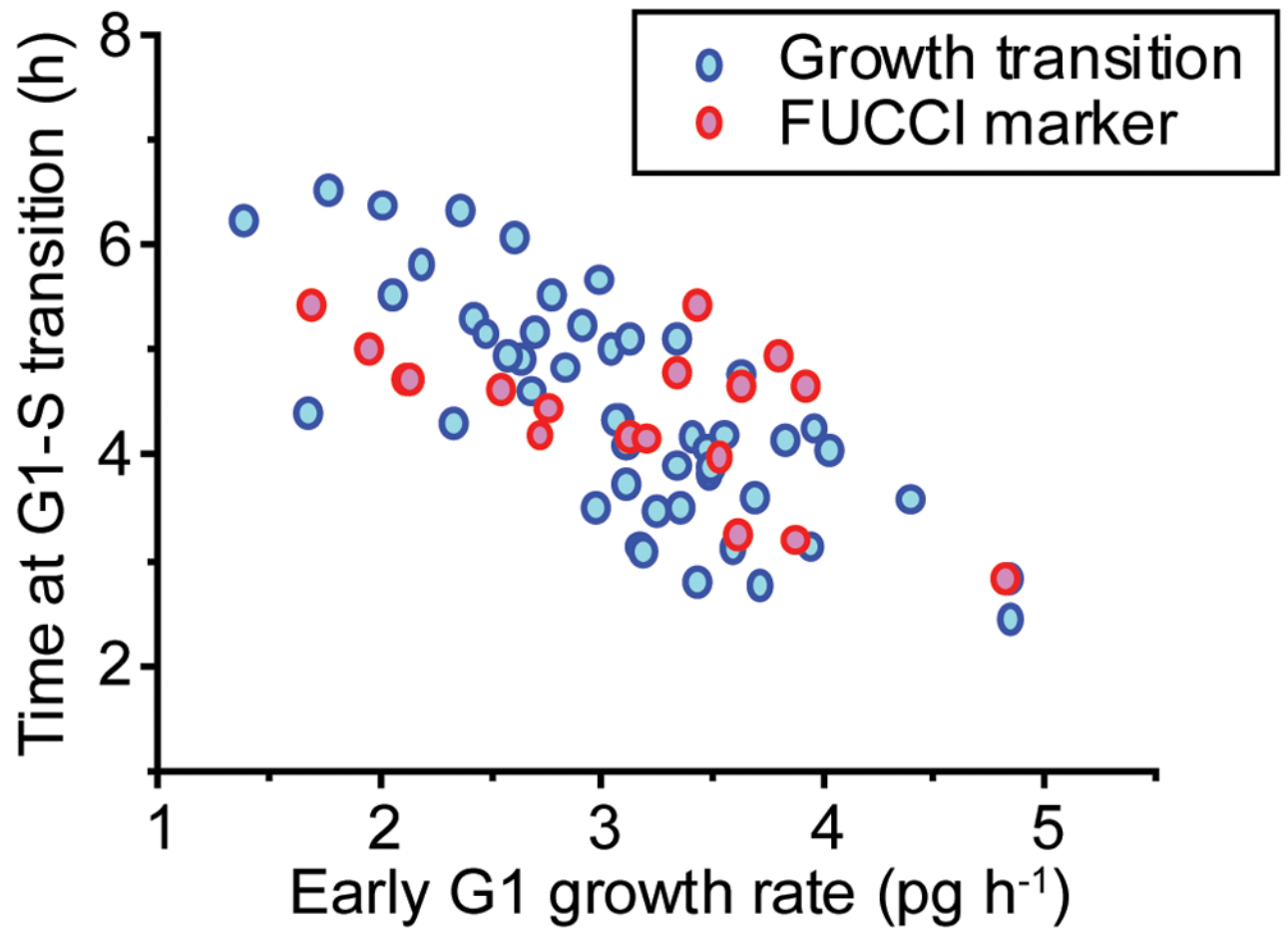


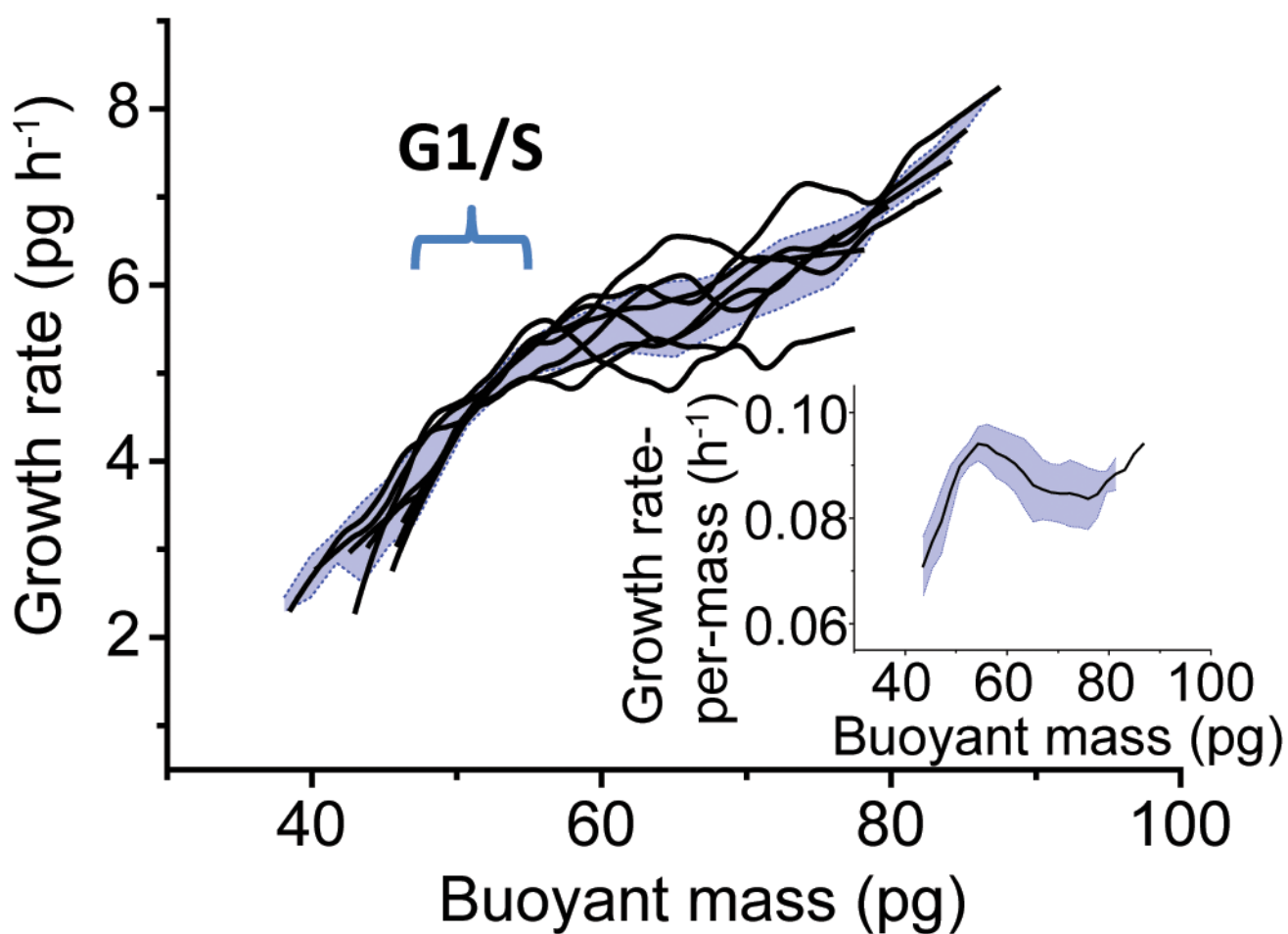
Figure 1.

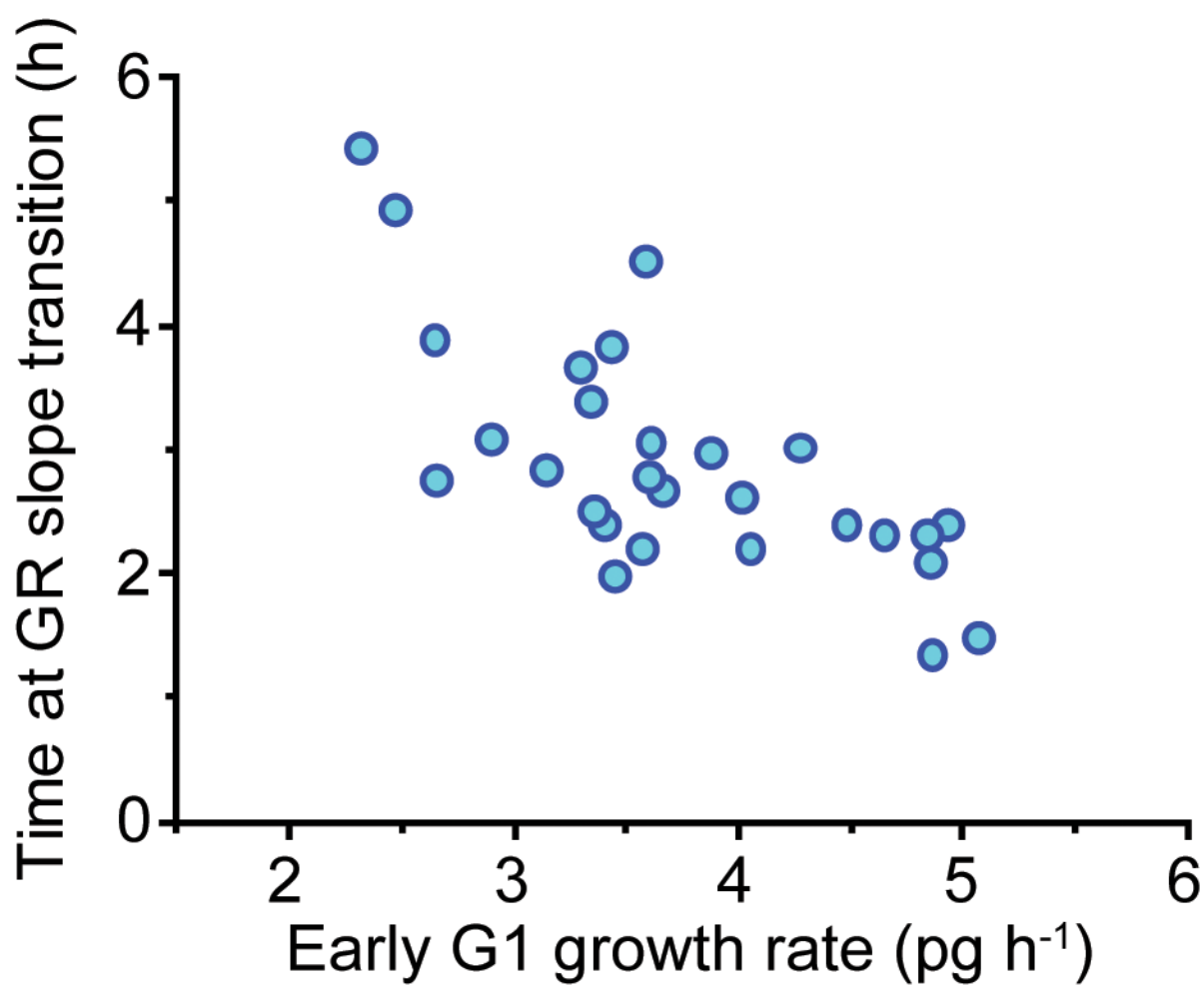
Measurement of single cell growth and cell cycle progression. **(a)** Pressure-driven flow (blue arrows) moves a cell between bypass channels and provides fresh media between measurements. The cell is weighed as it passes through the SMR (red path). Fluorescent signals are measured when the cell passes through the optical window in the bypass reservoir (yellow). **(b)** Mass (black, measured every 30 seconds) and fluorescent signals from the cell cycle reporters (blue – *cdt1* and red – *geminin*, measured every 30 minutes) were acquired from a L1210 mouse lymphoblast cell over four generations. The dashed box at the arrow zooms-in on signals from one transit through the SMR. Automated feedback between the mass signal and pressure regulators is used to transport the cell back-and-forth between the bypass channels. Following division, one daughter triggers the feedback while the other is swept away. **(c)** Growth rate versus cell mass obtained by measuring buoyant mass versus time of one cell from the newborn stage through division. Color bar indicates relative *cdt1* (red, G1 marker) and *geminin* (blue, S/G2/M marker) levels. Yellow indicates the G1 to S transition. Error bars at various cell sizes are determined by measuring the growth rate of a fixed (non-growing) cell over a 12 hr period and represent one standard deviation from zero growth rate. **Inset:** Correlation between the size at G1-S transition and

size at the growth slope transition ($n = 20$). Y-error bars indicate the mass change between the maximum and following minimum *cdt1* levels.









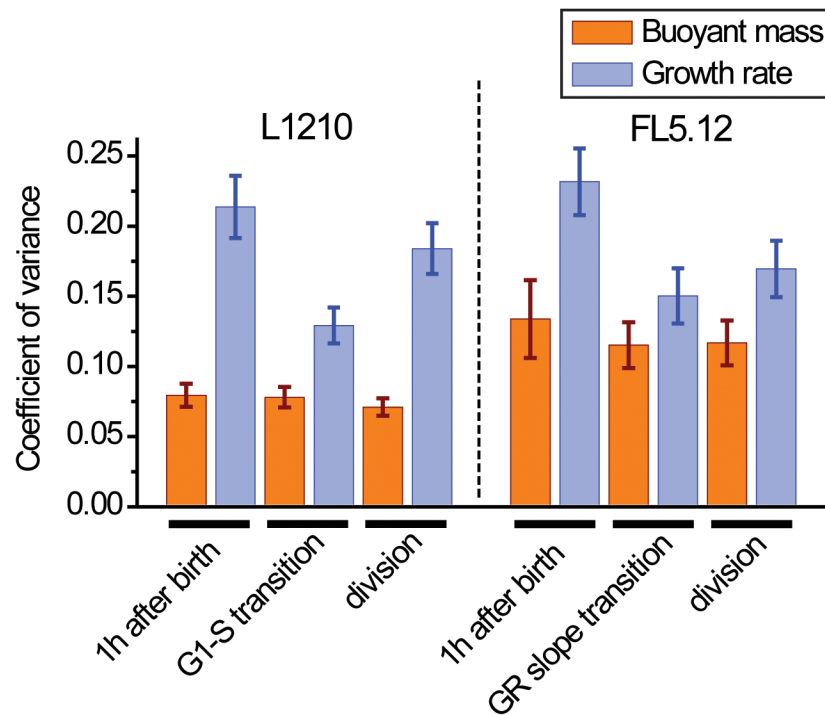


Figure 2.

Evidence for a growth rate threshold. **(a)** Single cell growth rate trajectories (black lines) for a lineage of five L1210 cells. Blue area defines five times the coefficient of variance. **Inset:** mean (black line) and standard deviation (blue area) of growth rate per unit mass from the lineage. **(b)** Time at G1/S phase transition versus early G1 growth rate (averaged between first and third hour of growth following division) for L1210 cells. Blue circles: G1/S transition defined by growth transition ($n = 49$, Pearson's correlation coefficient = -0.75). Red circles: G1/S transition defined by FUCCI ($n = 18$, Pearson's correlation coefficient = -0.60). **(c)** Single cell growth rate trajectories (black lines) for a lineage of eight FL5.12 cells. Blue area defines five times the coefficient of variance. **(d)** Time at G1/S phase transition versus early G1 growth rate for FL5.12 cells. G1-S transition is defined by calculating the growth transition point ($n = 28$, Pearson's correlation coefficient = -0.72). **(e)** Coefficient of variance (CV) for buoyant mass (orange) and growth rate (blue) at various points in the cell cycle. $n = 49$ for L1210 and $n = 28$ for FL5.12. Error bars represent one standard deviation of the CV from bootstrapping 1,000 times.